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NITROSATION OF CYSTEINE AND REDUCED GLUTATHIONE BY NITRITE AT PHYSIOLOGICAL PH

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1. ABSTRACT

Unlike the formation of nitrosothiols by nitrous acid, our study revealed that NO_2^- effectively reacted with L-cysteine or reduced glutathione (GSH) at pH 7.0 and 7.4, to form orange-pink products of S-nitrosocysteine (CySNO) or S-nitrosoglutathione (GSNO). The reactions were in a concentration-dependant manner. These products exhibited not only peak absorbances at around 340 and 540nm, but also unique colors and patterns of mobility on cellulose thin layer chromatographic plates. In comparison, the S-nitrosation of dithiothreitol was noted exclusively under acidic pH. In addition, the S-nitrosation of hemoglobin (Hb) by either peroxynitrite (PN) or NO_2^- at pH 6.0 was detected via Western blot. The half-life of degradation of CySNO in NO_2^- solution was significantly shorter than that of GSNO at a wide range of pH. In the absence of NO_2^-, degradation of GSNO was facilitated by incubation with L-cysteine, but not L-serine. In the signaling process involving NO $\rightarrow$ PN $\rightarrow$ NO_2^- $\rightarrow$ CySNO/GSNO $\rightarrow$ NO, L-cysteine may function as a NO-carrier to reach shorter-distance targets, and also an "activator" to release NO from GSNO. Furthermore, L-cysteine may play a vital role in reducing (severe) oxidative stress.

2. INTRODUCTION

Nitric oxide (NO) is an important signaling molecule (1-4), which can convert into peroxynitrite (PN), a potent oxidant (5-8). Both NO and PN can S-nitrosolate thiol and thiol-containing proteins (2,4,5,9,10). It was postulated that NO might react with oxygen to form the nitrosating agent N_2O_3 at pH 7.0, thereby producing S-nitrosothiol (9). S-Nitrosolation of protein thiols has been implicated in the NO-dependant regulation of many enzymes (11), including protein kinase C (12) and glyceraldehyde 3-phosphate dehydrogenase (13). S-Nitrosation may modulate the function of proteases, cytoskeletal proteins, membrane receptors (14), membrane ion channels (15), GTP-binding proteins (16), phosphotyrosine protein phosphatases (17), transcription factors (18,19) and glutathione reductase (20). Moreover, S-nitroso-serum albumin (21,22) has been proposed to act as an endogenous regulator of vessel tone (23). The occurrence of S-nitrosothiols in a variety of tissues may represent a novel class of signaling molecules (24), in addition to NO; its formation is catalyzed by constitutive and inducible nitric oxide synthase (NOS) (1,25). The over activation and/or over expression of NOS are responsible for the over production of NO and consequently attribute to various diseases. For example, the S-nitrosolation of matrix metalloproteinases may induce neuronal apoptosis and neurodegenerative disorders such as stroke, Alzheimer's disease, HIV-associated dementia, and multiple sclerosis (26-29). Nitric oxide and nitrous acid are interchangeable (1,2). Also, PN, a short-lived molecule, can degrade into NO_3^- and NO_2^- (30-32). Nitrite can be protonated to form nitrous acid in extremely acidic conditions (pH < 3), thereby nitrosolating thiols (1). Accumulation of NO_2^- was found in ischemic brain (26) and in human immunodeficiency virus infection (33). Furthermore, significant levels of NO_2^- can also enter the human body through dietary and respiratory intake (1) despite its unknown health consequence. It is important to further elucidate NO_2^--mediated pathogenesis, especially via the mechanisms of S-nitrosation under physiological pH. Hence, this investigation attempted to synthesize S-nitrosothiol directly from the reaction mixture of sodium nitrite with thiol, at pH 7.0 and 7.4 by the omission of strong acid, and subsequently analyze the degradation of S-nitrosothiol.

3. MATERIALS AND METHODS

3.1. Materials

L-Cysteine (HCl), L-serine, reduced glutathione (GSH), nitrosoglutathione (GSNO), dithiothreitol, sodium nitrate, sodium nitrite, sodium dodecyl sulfate (SDS), hemoglobin (Hb, from pig), cellulose thin layer

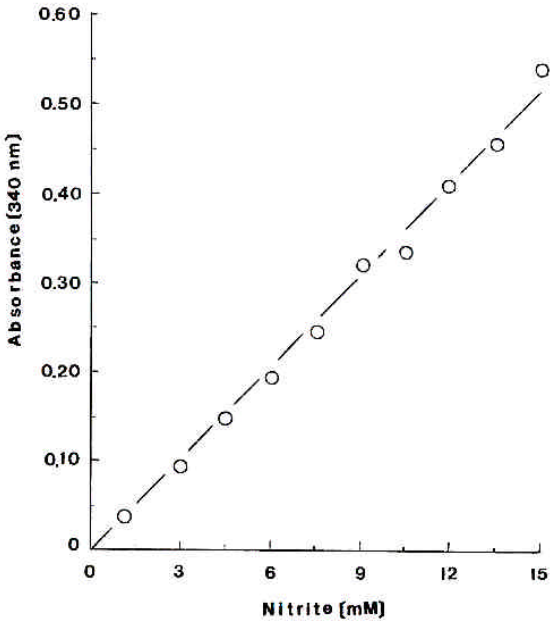

Figure 1. Concentration-dependence of NO_2^- on the formation of S-nitrosocysteine (CySNO). Incubation mixture was in a final volume of 300µl of 100mM potassium phosphate buffer (pH 7.0) containing 20mM L-cysteine and varied amounts of NO_2^-. The reaction was carried out at 25ºC for 5 minutes. The values presented had been corrected for the basal (control) values in the presence of NO_2^- alone.

chromatographic plates, all were supplied by Sigma Chemical Company, St. Louis, MO, USA. Western blotting reagents, nitrocellulose membranes and anti-rabbit IgG alkaline phosphatase conjugate were obtained from Bio-Rad Laboratories, Hercules, CA, USA. Active and degraded peroxynitrite (PN) were purchased from Upstate Biotechnology, Lake Placid, NY, USA. Rabbit polyclonal anti-S-nitrosocysteine was provided by Calbiochem-Novabiochem Corporation, San Diego, CA, USA. Other chemicals for thin layer chromatography solvent system preparation were acquired from Fisher Scientific, Pittsburgh, PA, USA.

3.2. Methods

Distilled/deionized H_2O, which may still contain trace metal cation, was used to prepare all solutions. The stock solutions of L-cysteine, L-serine, NO_3^-, NO_2^-, GSH and GSNO were all prepared in 200mM potassium phosphate buffer (pH 7.0 and 7.4). Prior to incubation, the buffer was not "deaired".

The products of the S-nitroso compound was monitored at 340 and 540nm using a Bio-Tek µQuant (MQX200) spectrophotometer microplate reader. If gas bubble production was too great for longer incubation periods, the samples were centrifuged at 13,500rpm for 1minute. Potassium phosphate buffer (pH 7.0 and 7.4) was used, and its pH was regularly monitored via electronic pH meter to ensure that buffer capacity was maintained (detailed- see legend of corresponding experiment).

With minor modifications, the experimental procedures of Western blot basically followed those from past studies (34,35). Bio-Rad's MiniPROTEIN 3 Cell was employed for both SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (36) and protein blotting (to the nitrocellulose membrane). Experiments required pre-cooled tank buffer and prefrozen Towbin transfer buffer. Anti-S-nitrosocysteine was used as the primary antibody (0.05µg/ml) (36), and anti-rabbit IgG-AP conjugate (37) as the secondary antibody.

S-Nitrosolated L-cysteine and GSH were also detected by cellulose thin layer chromatographs and developed in appropriate solvent systems (detailed- see legend of corresponding experiment). Before detection with ninhydrin spray and subsequent heating at 100°C, the plates were saturated in ammonium hydroxide vapor in a closed tank for 20 minutes.

4. RESULTS AND DISCUSSION

To avoid pre-protonation, buffers at acidic pH were not used. Instead, 1M potassium phosphate buffer at pH 7.0 was used to prepare nearly saturated sodium nitrite solution and L-cysteine solution, separately. After 5 minutes of mixing these solutions, an orange-pink color appeared. The higher concentration has facilitated the observation of the colored compound. The resulting product after being diluted to 130mM potassium phosphate buffer (pH 7.0) displayed peak absorbances at around 340 and 540nm (data not shown). However, when the pH of the buffer changed from 7.0 to 7.4, less intense color was observed. These initial findings provided the basis for the following detailed experiments.

Reaction of L-cysteine with sodium nitrite at pH 7.0 in 100mM potassium phosphate buffer resulted in an increase of net absorbance at 340nm, and it was in a NO_2^- concentration-dependant manner, from 1mM up to 15mM (Figure 1). The increase may be due to the production of CySNO. Nitrite alone exhibited a substantial absorbance at 340nm, but not at 540nm (data not shown). Therefore, the greater concentration of L-cysteine, 20mM, versus that of the maximal concentration of NO_2^-, 15mM, was required for the reaction to gain net absorbance. Accordingly, the net absorbance thus observed was corrected with the basal value of NO_2^- when present alone. The incubation of 30mM L-cysteine or GSH with 20mM NO_2^- yielded unique product(s) which exhibited peak absorbance around 340 and 540nm (Figure 2). The absorbance around 340nm was much more intense than that of 540nm (usually 10- to 20-fold). The formation of the orange-pink colored product and its peak absorbance are in agreement with those reported by Stamler and Feelisch (38). The pH of the incubation mixture was maintained at 7.0 or 7.4 by using 130mM potassium phosphate buffer throughout this experiment. Therefore, S-nitroso compounds were indeed generated at physiological pH in comparison with experiments under acidic conditions (4). The absorbance at 340 or 540nm at pH 7.0 was significantly greater than those at pH 7.4, indicating more S-nitroso was formed at pH 7.0. In another separate experiment, when 10mM L-cysteine or

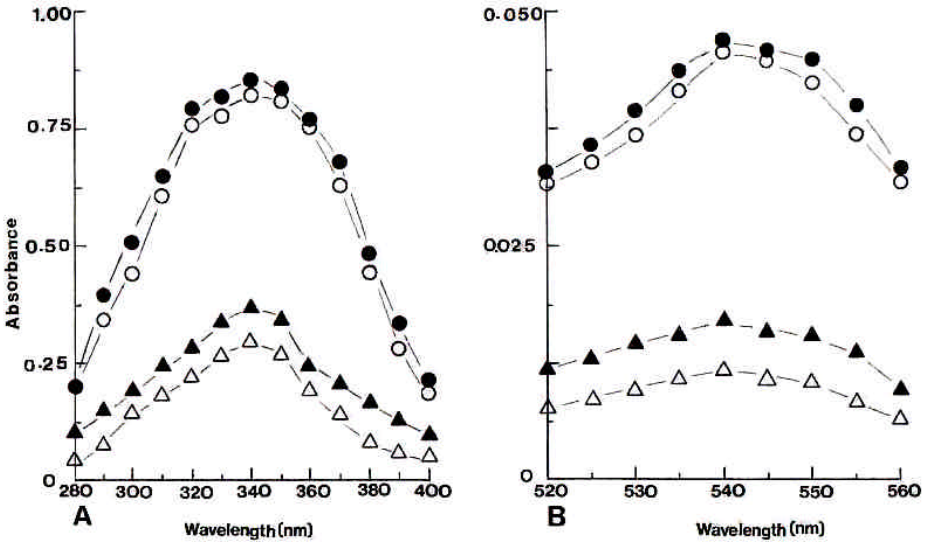

Figure 2. Absorption spectra of CySNO and S-nitrosoglutathione (GSNO) formed in NO_2^- solution. Incubation mixtures were in a final volume of 300µl of 130mM potassium phosphate buffer (pH 7.0 or 7.4) containing 20mM of NO_2^- and 30mM L-cysteine or GSH. The reactions were carried out at 25°C for 5 minutes and monitored at 340 (A) and 540nm (B). At pH 7.0, the incubation of L-cysteine and NO_2^- (●), or GSH and NO_2^- (○). At pH 7.4, the incubation of L-cysteine and NO_2^- (▲), or GSH and NO_2^- (△). The values presented had been corrected for the basal (control) values in the presence of NO_2^- alone.

GSH was incubated with 1mM NO_2^- in 50mM potassium phosphate buffer (pH 7.0), a significant net absorbance at 340nm was also observed (data not shown). It is noteworthy that significant amounts of NO_2^- and L-cysteine from dietary intake may preform nitrous acid and cysteine•HCl in gastric juice, thereby facilitating S-nitrosation of thiol either in the stomach or later on in the blood. Interestingly, during the incubation a significant amount of gas bubbles were produced. This may partially be due to the potassium phosphate buffer not being "deaired" prior to incubation. The composition of the gas evolved was unknown although it could be NO, O_2, CO_2, N_2O_4, N_2O_3, or others. When L-cysteine or GSH was replaced by L-serine, which contains a hydroxyl group, neither a significant change of the absorbance at 340 and 540nm nor the formation of an orange-pink product was noted, indicating that the thiol group is required for these reactions.

Under the same experimental conditions as in figure 2, the S-nitroso compound may degrade as indicated by the fading color and the decrease of the absorbance at 340nm during the progress of the reaction. At pH 7.0, the half-lives, as estimated by 50% decrease in absorbance, of formed CySNO and GSNO were observed at 1hr 36min and 2hr 55min, respectively. In comparison, at pH 7.4, the half-lives of formed CySNO and GSNO were noted at 1hr 30min and 3hr 25min, respectively (Figure 3). Following 4 hours of incubation, at pH 7.0 approximately 74% of CySNO and 56% of GSNO was degraded. In comparison, at pH 7.4 approximately 68% of CySNO and 58% of GSNO was degraded as estimated by the decrease of the absorbance. The shorter half-life of CySNO in NO_2^- solution may imply that CySNO is more capable of releasing NO than GSNO. Furthermore, the significantly shorter half-life of CySNO compared to that of GSNO was also observed using 130mM of various buffers, including phosphoric acid (pH 2.12), disodium hydrogen citrate (pH 5.40), tricine (pH 8.15) and boric acid (pH 9.24), though the wavelength of peak absorbance slightly varied (data not shown). The degradation of CySNO and GSNO may be due to the release of NO because the S-nitroso compound could be a NO-donor in aqueous solution (39). Surprisingly, exclusively under acidic conditions the incubation of dithiothreitol with NO_2^- also generates an orange-pink colored product. It may also be a S-nitroso compound because dithiothreitol contains thiol in addition to hydroxy groups. L-Cysteine exhibits pK1 (a-COOH), pKR (R group), and pK2 (a-NH_3^+) at 1.92, 8.33 and 10.78, respectively (40). Consequently, the thiol group and multiple pK values of L-cysteine may account for its versatility to form S-nitroso compounds at a wide range of pH.

In the absence of sodium nitrite, GSNO in potassium phosphate buffer displayed maximal absorbance around 340 and 540nm (Figure 4). When L-serine was incubated with GSNO, the instant but slight decrease in absorbance from 3.3 to 2.5 at 340nm and from 0.065 to 0.055 at 540nm for the incubation at pH 7.0, and from 3.4 to 2.6 at 340nm, and from 0.065 to 0.055 at 540nm for the incubation at pH 7.4 was noted. Similarly, the instant but slight decrease in absorbance was observed around 340 and 540nm when GSNO was incubated with L-cysteine. However, for further decrease in absorbance, GSNO must be incubated with L-cysteine, but not L-serine (Figure 5). During the incubation of GSNO with L-cysteine, the orange-pink color progressively faded, and the absorbance

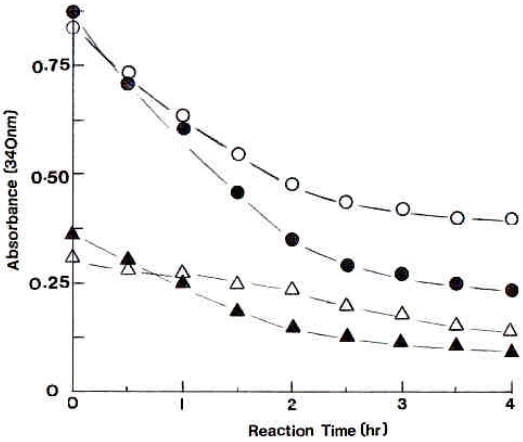

Figure 3. The degradation of CySNO and GSNO formed in NO_2^- solution. Incubation mixtures were in a final volume of 300µl of 130mM potassium phosphate buffer (pH 7.0 or 7.4) containing 20mM NO_2^- and 30mM L-cysteine or GSH. At pH 7.0, the incubation of L-cysteine and NO_2^- (●), or GSH and NO_2^- (❍). At pH 7.4, the incubation of L-cysteine and NO_2^- (▲), or GSH and NO_2^- (△). The values presented had been corrected for the basal (control) values in the presence of NO_2^- alone.

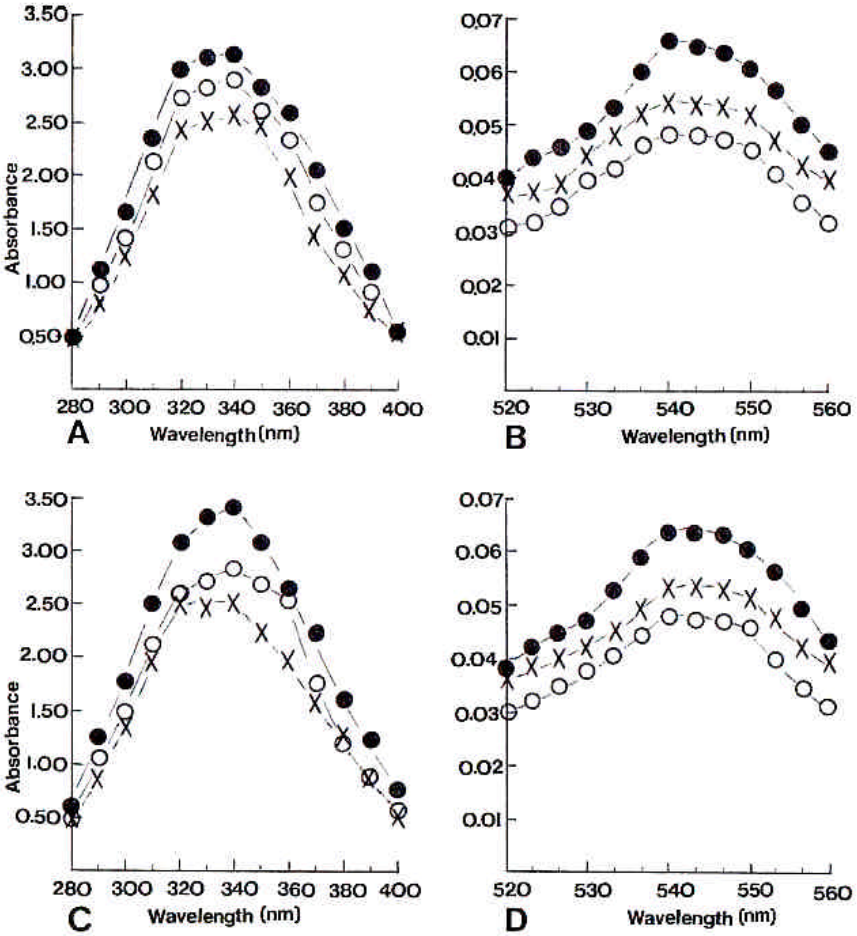

Figure 4. Absorption spectra of GSNO. Incubation mixtures were in a final volume of 300µl of 65mM potassium phosphate buffer (pH 7.0 or 7.4) containing 5mM of GSNO and L-cysteine or L-serine. The reactions were carried out at 25°C for 5 minutes and monitored at 340 (A at pH 7.0; C at pH 7.4) and 540nm (B at pH 7.0; D at pH 7.4). The incubation of 5mM GSNO alone (●), 5mM GSNO and L-cysteine (❍), or 5mM GSNO and 5mM L-serine (x).

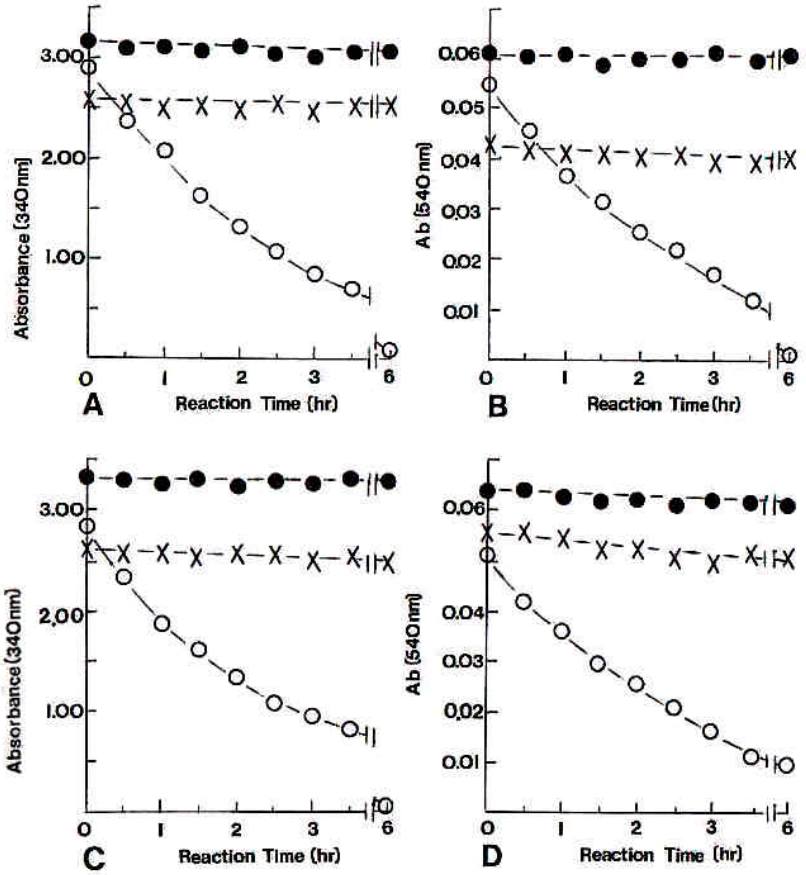

Figure 5. The degradation of GSNO. Incubation mixtures were in a final volume of 300µl of 65mM potassium phosphate buffer (pH 7.0 or 7.4) containing 5mM of GSNO and L-cysteine or L-serine. The decrease in absorbance was monitored at 340 (A at pH 7.0; C at pH 7.4) and 540nm (B at pH 7.0; D at pH 7.4). The incubation of 5mM GSNO alone (●), 5mM GSNO and L-cysteine (❍), or 5mM GSNO and 5mM L-serine (x).

decreased with a half-life of 1hr 55min at 340nm and 1hr 30min at 540nm for the incubation at pH 7.0, and 2hr 10min at 340nm and 1hr 50min at 540nm for the incubation at pH 7.4. Following 6 hours of incubation with L-cysteine, the absorbance for GSNO decreased 96% at 340 and 540nm for the incubation at pH 7.0, and 96% at 340nm and 81% at 540nm for the incubation at pH 7.4 (Figure 5). The results suggest that the degradation of GSNO may be due to the direct release of NO from GSNO and was enhanced by the presence of L-cysteine. Alternatively, transnitrosation of the NO-group from GSNO to L-cysteine may occur, followed by the rapid release of NO from CySNO. In contrast, the incubation of GSNO with L-serine resulted in no absorbance change or color fading within a 6-hour period (Figure 5), indicating neither the activation of NO release from GSNO, nor the transnitrosation of nitroso group from GSNO to L-serine occurred. Therefore, the thiol group of L-cysteine, but not the hydroxyl group of L-serine, may facilitate such degradation.

The formation of CySNO and GSNO, by incubation with NO_2^- was further analyzed by cellulose thin layer chromatography (Figures 6 and 7). Since peroxynitrite (PN) can degrade into NO_2^- (31), the incubation of degraded PN with GSNO or L-cysteine forms an orange-pink colored compound. When these incubated samples were developed in a solvent system containing 2-propanol: HCl: 2-butanone (180:72:45) (Figure 6A), L-cysteine alone (lane 1) exhibited less mobility/more tailing and a dark yellow color in the middle region, which was surrounded by a dark purple border; whereas, samples treated with degraded PN (lane 2) and active PN (lane 3) exhibited greater mobility/less tailing and a decreased dark yellow color in the middle region of the bands. This may be due to the S-nitrosolation of L-cysteine by both the degraded PN (which contains NO_2^-) and active PN. In addition, when the development was carried out with the solvent system containing methanol: chloroform (225:75) (Figure 6B), L-cysteine alone (lane 1) migrated away from its origin; whereas, degraded PN- and active PN-incubated mixtures (lanes 2 and 3) had more sample remaining at the origin. The finding suggests that L-cysteine may react with degraded PN and active PN and produce similar compounds, mainly CySNO.

After being developed in a solvent system containing 2-propanol: HCl: 2-butanone (180:72:45)(Figure 7), GSH alone and NO_3^--treated GSH (lanes 1 and 2)

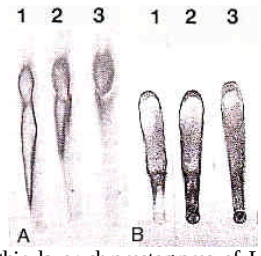

Figure 6. Cellulose thin layer chromatograms of L-cysteine and its nitrosolated products. Plates were developed with the solvent systems (A) 2-propanol:HCl:2-butanone (180:72:45) and (B) methanol:chloroform (225:75), and detected with ninhydrin spray. Incubation was in 50mM potassium phosphate buffer (pH 7.0). Approximately 1-2mg of L-cysteine samples were spotted. (A) Lane 1, L-cysteine (alone); Lane 2, L-cysteine incubated with 70µM degraded peroxynitrite (PN); Lane 3, L-cysteine incubated with 70µM active PN. (B) Lane 1, L-cysteine incubated with 30mM NO_2^-; Lane 2, L-cysteine incubated with 70µM degraded PN; Lane 3, L-cysteine incubated with 30mM NO_2^-.

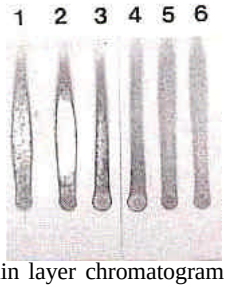

Figure 7. Cellulose thin layer chromatogram of GSH and its nitrosolated products. Plate was developed with the solvent system 2-propanol: HCl: 2-butanone (180:72:45), and detected with ninhydrin spray. Incubation was in 50mM potassium phosphate buffer (pH 7.0). Approximately 1-2mg of GSH samples were spotted. Lane 1, GSH (alone); Lane 2, GSH incubated with 30mM NO_3^-; Lane 3, GSH incubated with 70µM degraded PN; Lane 4, GSH incubated with 30mM NO_2^-; Lane 5, GSH incubated with 70µM of active PN; Lane 6, GSNO alone (2mg).

Figure 8. Effect of peroxynitrite (PN) (70µM)- and NO_2^- (0.15mM)-treatment on the S-nitrosation of pig hemoglobin (Hb) (30µg) detected by Western blot using anti-S-nitrosocysteine (0.05µg/ml). All samples were incubated in 50mM potassium phosphate solution (pH 6.0) at 25°C for 25min, and were subject to SDS-polyacrylamide gel electrophoresis (12%) prior to immunoblotting. Lane 1, Hb (alone); Lane 2, PN-Hb; Lane 3, NO_2^--Hb.

displayed similar colors and patterns in the chromatogram with white color in the middle surrounded by a purple border. These results imply that NO_3^- did not react with GSH, while degraded PN, which contains NO_2^- did (lane 3). Nevertheless, the conversion of NO_3^- into NO_2^-, and subsequent S-nitrosation *in vivo* cannot be excluded (1). Furthermore, the samples in lanes 2-5 displayed similar colors and patterns of migration as the pure form of GSNO (lane 6), suggesting that GSH, after reaction with degraded PN (lane 3), NO_2^- (lane 4) or active PN (lane5), produced GSNO (lane 6).

Our previous study (34) demonstrated that the addition of 1mM $FeCl_3$ and EDTA in the incubation mixture facilitated the S-nitrosation of hemoglobin (Hb) by PN at pH 7.0. However, the omission of $FeCl_3$ and EDTA in the incubation mixture resulted in the requirement of a higher concentration of PN (up to 70µM), and a decrease in pH to 6.0 to detect immunoreactivity of the CySNO residue of Hb (Figure 8, lane 1). One intense common band was observed at low molecular mass range for both PN-treated Hb (Figure 8, lane 2) and NO_2^--treated Hb (Figure 8, lane 3). Interestingly, another band with a large molecular mass was also noted in the PN-treated Hb, and it may be due to the impurity. It has been reported that, at high pO_2, endothelium derived NO is captured by heme-iron in R-state Hb, and the captured NO can then form SNO-Hb (1). Nevertheless, it remains unclear whether the commercially obtained Hb used in this study had been denatured/deoxygenated, and whether such processes resulted in less S-nitrosolation. In addition to Hb, the possible S-nitrosolation on other proteins at physiological pH is still under investigation.

The sufficient uptake of L-cysteine (41), which is a precursor of GSH, may be crucial to maintain cellular homeostasis, and such significance was further supported by the results of this study. At physiological pH, the formation of CySNO and GSNO by incubation with NO_2^- and an even faster degradation of CySNO suggest that both L-cysteine and GSH may function as a NO-carrier in the related signaling process, despite shorter-distance target(s) for CySNO. Additionally, activation of the release of NO from GSNO by L-cysteine may be an effective mechanism to replenish cellular GSH. Accordingly, the formation of CySNO may also play a vital role in reducing the severe oxidative stress in which excessive NO, PN and NO_2^- are produced.

5. ACKNOWLEDGEMENT

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